PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : C12N 15/19, C07K 14/52, 16/24, A61K 38/19, 39/395, C12N 15/11, 15/70, 1/21 — A3

(11) International Publication Number: WO 96/09389

(43) International Publication Date: 28 March 1996 (28.03.96)

(21) International Application Number: PCT/SI95/00022

(22) International Filing Date: 18 September 1995 (18.09.95)

(30) Priority Data:
P-9400363 19 September 1994 (19.09.94) SI

(71)(72) Applicant and Inventor: MOZETIČ FRANCKY, Bojana [SI/SI]; Gorazdova 7, 61000 Ljubljana (SI).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): FRANCKY, Andrej [SI/SI]; Gorazdova 7, 61000 Ljubljana (SI).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

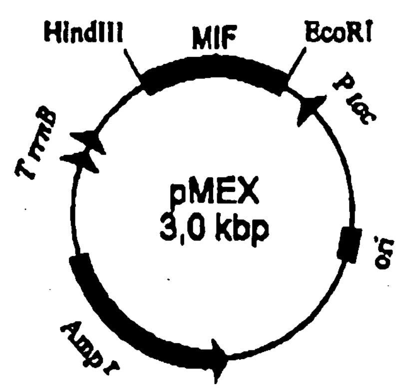
(88) Date of publication of the international search report: 23 May 1996 (23.05.96)

BEST AVAILABLE COPY

(54) Title: RECOMBINANT MACROPHAGE MIGRATION INHIBITORY FACTOR: EXPRESSION IN ESCHERICHIA COLI AND PURIFICATION OF RECOMBINANT PROTEIN

(57) Abstract

According to the known cDNA sequence we used the PCR (polymerase chain reaction) technique to isolate a gene encoding human MIF from uterus endometrium The PCR product was cloned in the *Escherichia coli* plasmid vector pUC 19 and the nucleotide sequence was confirmed. We were able to successfully express human MIF in the bacteria *Escherichia coli* by use of the pKP 1500 expression plasmid. The recombinant protein accumulated intracellularly in soluble form, comprising more then 30 % of total cell protein. We have designed an original two step procedure where protein purification was accomplished by gel filtration and ion exchange chromatography. The purified recombinant MIF was used to immunise a rabbit and antibodies obtained were used for MIF detection in human tissues by immunohistochemical techniques.



08/243,342 7815-008

REF. CB

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/19, C07K 14/52, 16/24, A61K 38/19, 39/395, C12N 15/11, 15/70, 1/21

A2

(11) International Publication Number: WO 96/09389

(43) International Publication Date: 28 March 1996 (28.03.96)

(21) International Application Number: PCT/SI95/00022

(22) International Filing Date: 18 September 1995 (18.09.95)

(30) Priority Data:
P-9400363 19 September 1994 (19.09.94) SI

(71)(72) Applicant and Inventor: MOZETIČ FRANCKY, Bojana [SI/SI]; Gorazdova 7, 61000 Ljubljana (SI).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): FRANCKY, Andrej [SI/SI]; Gorazdova 7, 61000 Ljubljana (SI).

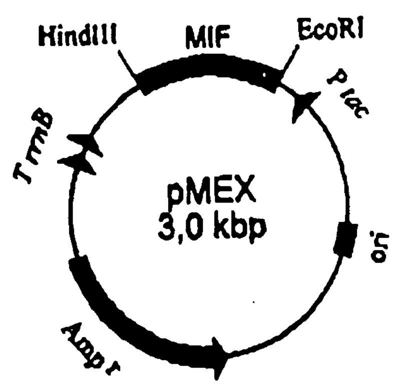
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT MACROPHAGE MIGRATION INHIBITORY FACTOR; EXPRESSION IN ESCHERICHIA COLI AND PURIFICATION OF RECOMBINANT PROTEIN

(57) Abstract

According to the known cDNA sequence we used the PCR (polymerase chain reaction) technique to isolate a gene encoding human MIF from uterus endometrium The PCR product was cloned in the *Escherichia coli* plasmid vector pUC 19 and the nucleotide sequence was confirmed. We were able to successfully express human MIF in the bacteria *Escherichia coli* by use of the pKP 1500 expression plasmid. The recombinant protein accumulated intracellularly in soluble form, comprising more then 30 % of total cell protein. We have designed an original two step procedure where protein purification was accomplished by gel filtration and ion exchange chromatography. The purified recombinant MIF was used to immunize a rabbit and antibodies obtained were used for MIF detection in human tissues by immunohistochemical techniques.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/SI 95/00022

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9011301	04-10-90	CA-A-	2050332	18-09-90
		EP-A-	0463037	02-01-92
		JP-T-	4504111	23-07-92
WO-A-9219727	12-11-92	US-A-	5328990	12-07-94
		AU-B-	1990992	21-12-92
WO-A-9426307	24-11-94	AU-B-	6834594	12-12-94
		CA-A-	2163211	24-11-94

RECOMBINANT MACROPHAGE MIGRATION INHIBITORY FACTOR; EXPRESSION IN ESCHERICHIA COLI AND PURIFICATION OF RECOMBINANT PROTEIN

BACKGROUND OF THE INVENTION

The macrophage migration inhibitory factor (MIF) was the first lymphokine to be discovered. In 1966 it was shown that antigen stimulated lymphocytes produced a soluble factor that inhibited the migration of macrophages in vitro (Bloom, B. R. & Bennett, B. (1966) Science 153, 80-82 / David, J. R. (1966) Proc. Natl. Acad. Sci. USA 65, 72-77). MIF containing supernatants of stimulated lymphocytes were subsequently shown to alter macrophage functions and enhance the killing of micro-organisms and tumour cells (Churchill, W. H. , Piessens W. F. , Sulis, C. A. , David, J.R. (1975) J. Immunol. 115, 781 / Nathan, C. F., Karnovsky, M.L., David J. R. (1971) J. Exp. Med. 133, 1356 / Nathan, C. F., Remold, H. G., David, J. R. (1973) J. Exp. Med. 137, 275). MIF has also been shown to correlate with delayed type hypersensitivity and cellular immunity (Bloom, B. R. & Bennett, B. (1966) Science 153, 80-82 / David, J. R. (1966) Proc. Natl. Acad. Sci. USA 65, 72-77 / David, J. R. & David, R. A. (1972) Prog. Allergy 16, 300-449) MIF activity has been detected in the synovia of patients with rheumatoid polyartritis (Odink, K. , Cerletti, N., Bruggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R. & Sorg, C. (1987) Nature (London) 330, 80-82), in leukocyte culture supernatants of mice during allograft rejection (Al-Askari, S., David, J. R., Lawrence, H. S. & Thomas, L. (1965) Nature (London) 205, 916-917 / Harrington, J. T. (1977) Cell. Immunol. 30, 261-271) and in various chronic inflammatory loci (Burmeister, G., Zwadlo, G., Michels, E., Brocker, E . & Sorg, C. (1984) Lymphokine Res. 3, 236 (abstr.), Schlegel-Gomez, R. ,

Diepgen, T. L. , Neumann C. , Sorg, C. (1990) Arch. Dermatol. Res. 282, 374-378

However, a purified or cloned lymphokine was required to demonstrate that these altered macrophage functions were induced by MIF and not by any other factor. Low levels of activity expressed by natural sources proved the biochemical characterisation of MIF very difficult, since the native protein has not been isolated in sufficient amounts yet.

The cDNA encoding human MIF was cloned in COS cells in 1989 by Weiser et al.. By functional expression cloning of the cDNA from T cells a clone was identified which expressed a strong MIF activity. Waiser also stressed that MIF is the product of activated lymphocytes T only. (Weiser, W. Y., Temple P. A., Witek-Giannotti J. S., Remold, H. G., Clark, S.C. & David, J.R. (1989) Proc. Natl. Acad. Sci. USA 86, 7522-7526). Supernatants from COS cells (bearing a recombinant gene encoding human MIF) were shown to stimulate the antibody synthesis (Weiser, W. Y., Pozzi, L. M. , David J. R. & Titus, R. G. (1992). Proc. Natl. Acad. Sci. 89, 8049-8052). Preliminary studies on *Leischmania donovani* (intracellular parasite) infections (Weiser, Y. W. , Pozzi, L. M. & David J. R. (1991) J. Immunol. 147, 2006-2011) and the demonstration that MIF containing supernatants from recombinant COS cells have the ability to activate macrophages to express nitric oxide synthetase and to produce NO (Cunha, F. Q., Weiser W. Y., David, J. R., Moss D. W., Moncada, S. & Liew, F.Y. (1993) J. Immunol. 150, 1908 -1912 / Liew, F. Y. ,Millot, S. , Parkinson, C. , Palmer, R. M. J. , Moncada, S. (1990) J Immunol. 129, 351) indicated that the newly discovered (12 kDa) protein probably acts as a critical factor in cell mediated immune host defences . Recently it was reported that MIF plays a central role in the toxic response to endotoxemia and possibly toxic shock (Bernhagen, J., Calandra, T., Mitchell, R.A., Martin, S. B., Tracey K., J., Voelter W., Manogue K. R., Cerami, A. & Bucala R. (1993) Nature 365, 756-759). MIF was isolated from ocular lens by Wistow et al. in 1993 (Wistow G. J., Shaughnessy, M. P., Lee,

D. C., Hodin, J. & Zelenka, P. S. (1993) Proc. Natl. Acad. Sci. USA 90, 80490-80529) and a putative MIF from rat liver was isolated by Blocki et al. in 1992 (Blocki, F. A., Schlievert, P. M. & Wackett, L.P. (1992) Nature 360, 269-270). This protein that matches the primary structure of a human leukocyte MIF (Weir, 1989) in 25 out of 26 terminal amino acids and which has both glutathione transpherase and MIF activity was shown to link chemical and immunological detoxification systems. Suzuki and colleaques (Suzuki, M. , Murata E. & Tanaka I. (1994). J. Mol. Biol. 235, 1141-1143) reported about crystallisation and preliminary crystallographic studies of MIF from human lymphocytes. From this article it can be realised that human MIF was expressed in E. coli and purified by affinity chromatography on the basis of the results of Blocki et al. (Blocki, F. A., Schlievert, P. M. & Wackett, L.P. (1992). Nature 360, 269-270)

Although the focus of research and hence the need for this protein continues to expand and despite the fact that MIF from natural sources has never been isolated in sufficient amounts yet and that the quantity of recombinant protein produced by tissue cultures is low, the information about expression of MIF in bacteria are very scarce. The data about the yields are generally not reported and the purification procedures are only mentioned and not well described. Some purification procedures include affinity chromatography based on glutathione transferase activity of MIF. The identity of purified proteins, isolated by affinity chromatography, was however not checked by N-terminal amino acid sequencing although it should be since glutathione transferase activity was not confirmed for human MIF and is sometimes controversial. In addition, the above mentioned affinity chromatography procedure could not be specific for recombinant protein (bacterial proteins with glutathione affinity could also be isolated).

On the other hand, as we know, no MIF protein is commercially available, although human cDNA is marketed by some companies.

SUMMARY OF THE INVENTION

The present invention relates to the construction of an expression vector for human MIF in *Escherichia coli* and the establishment of an original optimised protocol that enable to express and isolate large amounts of highly purified and biologically active recombinant human MIF. From 50 grams of recombinant bacterial cells (wet weight) 1 gram of highly purified and biologically active protein can be obtained. In this way various biochemical, biophysical and physiological studies of this still poorly understood cytokine should be facilitated. MIF could have also specific therapeutic and diagnostic values. From this point of view it is still more important to obtain it in large quantities and in a pure, biologically active form.

Purified recombinant MIF could serve to produce MIF specific antibodies which could be used to detect MIF in various human tissues by immunohistochemical methods. We clearly demonstrated the presence of MIF in epithelial cells of some samples of human uterus endometrium (where it is probably connected with certain pathologic states), in epithelial cells of various inflammatory tissues (human and animal) and in the same cell type in allograft rejections of human kidney. Kidney epithelial cells were almost completely negative in healthy individuals.

In this way MIF and (or) against MIF directed antibodies could be used as diagnostic or prognostic markers.

The present invention thus relates also to MIF that is expressed in epithelial cells. Besides its general function in the immunological system, the above described discoveries opened also the possibility that MIF acts as an important factor in mucosal immunity. Epithelial cell MIF could be a

signal for injury, infection, tissue invasion, ect. and could serve as a chemoatractant and activator of macrophages (and possibly other immune cells) on the site of inflammation. In this sense it could serve as one of the first cytokines in the complex cytokine network.

MIF alone or in combination with other moleculas (such as IFN-γ, IL-2, ect.) could be used to treat diseases where cellular / mucosal immunity should be stimulated (such as infections, AIDS, cancer, etc.). On the other hand, against MIF directed antibodies or MIF antagonists could be used when immunological functions should be scaled down (like in the case of auto immune diseases, tissue or organ transplantations, etc.).

The present invention is thus directed to purified recombinant MIF, to its analogues, MIF specific antibodies and antagonists that could be (in any pharmaceutical combination) used as diagnostic, prognostic or therapeutic markers/agents.

DETAILED DESCRIPTION OF THE INVENTION

Amplification by polymerase chain reaction (PCR) on a cDNA template could be used to isolate the MIF coding region and to create restriction sites which enable to express MIF in different *Escherichia coli* expression vectors. DNA amplification products could be analysed by gel electrophoresis. Vectors, such as pKP 1500 could be used to express the recombinant protein in the cytoplasm of E. coli cells. Others, such as pIN-III-omp A2 (containing a signal sequence) could be used for transporting MIF into the E. coli periplasmic space.

The efficiency of the expression due to different expression vectors (and E. coli strains) could be determined by protein analyses of whole cell extracts (lysates). The cell extracts (lysates) are prepared as described in example 1 and analysed by SDS-PAGE and IEF using known mol. wt. or pI

standards and a control (a total cell lysate or crude extract of host E. coli cells, bearing the expression vector without insert).

A strong new band (corresponding to an approximately 12 kDa protein) appears at the expected position when E. coli cultures with recombinant pKP 1500 plasmid, harbouring MIF insert are analysed.

According to the results of SDS-PAGE E. coli YM 109 bearing the recombinant pKP 1500 plasmid (designed pMEX) and expressing MIF intracellularly could be selected for large scale production (fermentation) and subsequent purification of the protein. All purification steps should be done at 4°C to minimise proteolysis. Gel filtration chromatography could be used as a first purification step. The aim of this choice is to remove very large molecules (nucleic acids such as plasmids and chromosomal DNA, high molecular proteins, etc. and very small ones (such as many bacterial toxins and pyrogens, culture media residual ingredients, ect.). On the other hand eventual soluble multimeric molecules, resulted of improperly folded recombinant protein, could be eliminated in this way. Purification of the product could be monitored by SDS-PAGE and IEF that produced bands at expected positions according to the known mol. wt. and pI standards and in the comparison with the control. Fractions which are found to be reach in recombinant protein are pooled, concentrated and further purified by ion exchange chromatography. Different buffers at different pH could be used at this purification step. A successful approach is when 10 mM phosphate buffer (pH 6,4) is used in combination with a CM-cellulose ion exchange column. This has also a logical explanation since the isoelectric point (pI) of recombinant human MIF is approximately 7,0 and bacterial proteins are predominantly more acidic. By using the phosphate buffer mentioned above it could happen that the recombinant protein did not bind to the CM cellulose. In this case it is recommended to adjust the pH

very carefully or even lower the pH for 0.1 or 0,2 units. The elution diagram of the purification by classical cation exchange chromatography on a CM cellulose column, using phosphate buffer pH 6,4, shows two peaks only. The first one represents unbound impurities (without recombinant protein), the second one is highly purified recombinant human MIF.

We have successfully expressed the human macrophage migration inhibitory factor (MIF) in *Escherichia coli* by use of the pKE 1500 expression plasmid containing the tac promoter and a temperature sensitive origin of replication, ensuring a high plasmid copy number at elevated temperatures. The recombinant protein accumulated intracellularly in soluble form, comprising more than 30% of total cell protein. We have designed a two step procedure where protein purification was accomplished by gel filtration on Sephacex G-50 and cation exchange chromatography on CM cellulose columns. The 12 kDa protein was shown to be pure by SDS-PAGE, IEF and by HPLC. The identity of the purified protein was verified by amino acid analyses and N-terminal sequencing. The MIF assay was used to measure its activity. 1 gram of highly purified and biologically active recombinant human MIF was obtained from 50 grams of *E.coli* cells (wet weight). CD spectra in the near UV and NMR analysis confirmed an ordered, native like structure of purified recombinant MIF.

During developing the purification procedure attention was given particularly to two problems. Firstly, isolation of soluble recombinant proteins from whole cell lysates as opposed to isolation of inclusion bodies or proteins from periplasmic space is more laborious. It is difficult to remove bacterial debris by filtration or centrifugation due to high viscosity. In this case the disruption of cells by French Press was proved unsuccessful. If cells are only partially broken by freezing and thawing only, following the procedure described in example 1, problems with viscosity are overcomed and this represents also a first purification step.

The second problem are the activity measurements. MIF assay is a biological test and hence laborious, time consuming and with lower reproducibility. On the other hand many factors (such as impurities in the sample) could contribute to the results of the test. So the activity measurements are not a good choice for MIF detection during the purification procedure. The pI of the recombinant MIF (approximately 7,0) is very different in comparison to bacterial proteins which are predominantly acidic (with pH values below 6,4). After isoelectric focusing of a control (total protein extract of E. coli YM 109 cells bearing the expression vector pKP1500 without insert) there are no bands at the expected position (pI higher than 6,4). This fact and excellent expression of the recombinant MIF permit the design of a purification procedure where all steps could be followed on the basis of IEF analyses of fractions without activity measurements.

We would like to stress that the excellent expression is not due only by the expression vector chosen (pKP 1500), but it is also the result of bacterial cell cultivation. Due to cultivation conditions the cells could be kept as long as needed in a highly repressive state regarding the production of recombinant MIF. The way of the preparation of the seed culture could be in this case very important since when growing in certain complex media, the cells could produce the recombinant protein even if they are not induced. Derepression during cell growth can have negative influences on biomass production and hence on the final yields of recombinant protein.

The procedure is specially adopted for large scale production and purification using classical methods. However it does not mean that quick purification methods with small capacities (such as FPLC) could not be used. If the concentration of endotoxins in the end product (purified MIF) is too high, additional purification steeps (such as reverse-phase chromatography) could be used. However, this is not necessary if the roles of aseptic work are respected.

Purified recombinant MIF served to produce MIF specific antibodies which we used to detect MIF in various human tissues by imunohistochemical methods. We clearly demonstrated its presence in epithelial cells of some samples of human uterus endometrium (where it is probably connected with certain pathologic states), in epithelial cells of various inflammatory tissues (human and animal) and in the same cell type in allograft rejections of human kidney. Kidney epithelial cells were almost completely negative in healthy individuals.

EXAMPLE 1

Construction of expression vectors

The coding region of MIF DNA was isolated by polymerase chain reaction (PCR). Double stranded cDNA that was used as a template for PCR was synthesised using mRNA from human uterus endometrium or from epithelial origin cultured cells and chemicals supplied by Amersham (cDNA synthesis kit). The reactions were done according to the Amersham instructions. mRNA was isolated by affinity chromatography on oligo dt spun columns (Sambrook, J., Maniatis, T. & Fritsch, E. F. Molecular Cloning: A laboratory manual; Cold Spring Harbor Laboratory (1989). The guanidinium isothiocianate method followed by isopicnic ultra centrifugation in a gradient of caesium trifluoroacetate was used for total RNA isolation .

The primers used were:

I. : 5' GGATCC<u>GAATTC</u>**ATG**CCGATGTTCATCGTAAACACCA 3'
(sense strand oligonucleotide corresponding to the N terminus of MIF ; Eco RI cutting site underlined and start codon in bold)

II.: 5' GTCGACAAGCTTTTAGGCGAAGGTGGAGTTGTTCCA 3'
(antisense strand oligonucleotide corresponding to the C terminus of MIF ; Hind III cutting site underlined and stop codon in bold)

The same primers in the same PCR were used to create Eco RI and Hind III cutting sites, flanking the coding region that allowed directional cloning into multiple cloning sites of E. coli vectors pUC 19 (Messing, J. (1983) Methods in Enzymology, Vol. 101, pp. 20-78 (Wu, R., Grossman, L. & Moldave, K., Ed.), San Diego:Academic Press), pIN-III-ompA2 (Auerswald, E. A. , Genenger, G. , Mentele, R. , Lenzen, S. , Assfalg-Machleidt, I. Mitschang, L. , Oschkinat, H. & Fritz H. (1991). Eur. J. Biochem., 200, 131-156 / Ghrayeb, J. ,Kimura, H. ,Takahara, M. , Hsiung, H., Masui, Z. , Inouye, M. (1984) EMBO J., 3 (10), 2437-2442) and pKP 1500 (Miki, T., Yasukochi, T., Nagatani, H., Furuno, M., Orita, T., Yamada, H., Imoto, T., Horiuchi, T. (1987) Protein Engineering 1, 327-332). Oligonucleotide synthesis was carried out using an Applied Biosystem DNA synthesiser, according to the manufacturer recommendations. The products were cleaved from columns and de protected by saturated ammonium hydroxide and further purified by polyacrylamide gel electrophoresis.

The cycling conditions for the polymerase chain reaction were denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. 100 µl of the reaction mixture contained: 10 mM Tris (pH 8,4), 50 mM KCl, 100 ng cDNA (incubated in a boiling water bath for 10 minutes prior added to the reaction mixture),1 µM oligonucleotide I,1µ M oligonucleotide II, deoxynucleotide triphosphates (dATP, dCTP, dGTP dTTP ; 200 µ M concentration each), 1mM $MgCl_2$, 2,5 units of Taq polymerase (Perkin Elmer) ; 100 µl of mineral oil was added on the top to prevent evaporation. A total of 30 PCR cycles were carried out in an Perkin Elmer thermo cycler. Terminal extension was allowed by heating the PCR product at 72°C for 10 minutes. Mineral oil was then carefully removed and following fenolisation and

ethanol precipitation (Sambrook, J., Maniatis, T. & Fritsch, E. F. Molecular Cloning: A laboratory manual; Cold Spring Harbor Laboratory (1989), restriction of the approximately 400 base pair fragment with EcoRI and Hind III endonucleases was performed for at least five hours under standard conditions (Sambrook, J., Maniatis, T. & Fritsch, E. F. Molecular Cloning: A laboratory manual; Cold Spring Harbor Laboratory (1989). The reaction mixture was then again fenolised, DNA was ethanol precipitated, dried in a speed-vac concentrator, resuspended in 50 µl of TE buffer and purified by gel filtration chromatography (1 ml Sephacryl S-300 spin columns). The E. coli plasmid vectors were digested and purified in the same way; except that Sephacryl S-400 was used in the gel filtration chromatography step. Inserts were ligated to vectors in a molar ratio 3 :1 in favour of inserts and the ligation reaction was performed at 15ºC for 15 hours under otherwise standard conditions

A fluorescent method for quantifying ng amounts of DNA was used to measure the concentration of vector and insert DNA (Sambrook, J., Maniatis, T. & Fritsch, E. F. Molecular Cloning: A laboratory manual: Cold Spring Harbor Laboratory (1989)). 1 µl of the ligation mixture was used to transform E. coli DH5α competent cells (Hannahan, D. (1983) Mol. Biol. 166, 557-580, Hannahan, D. (1985) Techniques on transformation of E.coli. In: DNA cloning: a practical approach, Vol.1, pp109-135 (Glover, D. M. Ed.). Oxford: IRL Press). Eighteen of the resulting clones were picked up and amplified in 3 ml of LBA medium. Plasmids were then isolated (Del Sal, G.; Manfioletti G. & Schneider C. (1988) Nucleic Acid. Res. 16, 9878) and MIF positive clones were identified by restriction analyses. The nucleotide sequences of four positive clones from three independent PCR were confirmed by the dideoxy sequencing method (Sanger, F., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA. 74, 5463).The nucleotide sequence and the deduced amino acid sequence of the sequenced clones are shown below

ATG CCG ATG TTC ATC GTA AAC ACC AAC GTG CCC CGC GCC TCC GTG 45

Met Pro Met Phe Ile Val Asn Thr Asn Val Pro Arg Ala Ser Val

5 10 15

CCG GAC GGG TTC CTC TCC GAG CTC ACC CAG CAG CTG GCG CAG GCC 90

Pro Asp Gly Phe Leu Ser Glu Leu Thr Gln Gln Leu Ala Gln Ala

20 25 30

ACC GGC AAG CCC CCC CAG TAC ATC GCG GTG CAC GTG GTC CCG GAC 135

Thr Gly Lys Pro Pro Gln Tyr Ile Ala Val His Val Val Pro Asp

35 40 45

CAG CTC ATG GCC TTC GGC GGC TCC AGC GAG CCG TGC GCG CTC TGC 180

Gln Leu Met Ala Phe Gly Gly Ser Ser Glu Pro Cys Ala Leu Cys

50 55 60

AGC CTG CAC AGC ATC GGC AAG ATC GGC GGC GCG CAG AAC CGC TCC 225

Ser Leu His Ser Ile Gly Lys Ile Gly Gly Ala Gln Asn Arg Ser

65 70 75

TAC AGC AAG CTG CTG TGC GGC CTG CTG GCC GAG CGC CTG CGC ATC 270

Tyr Ser Lys Leu Leu Cys Gly Leu Leu Ala Glu Arg Leu Arg Ile

80 85 90

AGC CCG GAC AGG GTC TAC ATC AAC TAT TAC GAC ATG AAC GCG GCC 315

Ser Pro Asp Arg Val Tyr Ile Asn Tyr Tyr Asp Met Asn Ala Ala

95 100 105

AAT GTG GGC TGG AAC AAC TCC ACC TTC GCC TAA 360

Asn Val Gly Trp Asn Asn Ser Thr Phe Ala *

110 115

With the exemption of one codone only, resulting in all cases in the same amino acid change, the sequences were identical as reported (Weisser, W. Y., Templc P. A., Witek-Giannotti J. S., Remold H. G., Clark S.C. & David J.R. (1999)

Proc. Natl. Acad. Sci. USA 86, 7522-7526). The amino acid substitution was exactly the same as found by Wistow (Wistow G. J., Shaughnessy, M. P., Lee, D. C., Hodin, J. & Zelenka, P. S. (1993) Proc. Natl. Acad. Sci. USA 90, 8049-8052)). At this moment in the literature exists a great confusion between cDNA/proteins with very similar nucleotide/amino acid sequences and between the biological functions these cDNA products/proteins could express.

One of the recombinant puC 19 plasmids, harbouring the MIF insert was Eco RI and Hind III digested and fractionated by gel electrophoresis. The band containing DNA of 400 base pairs was excised and isolated from the gel slice by adherence to glass powder (Vogelstein, B. & Gillespie, D. (1979) Proc. Natl. Acad. Sciu. USA 76, 615-619). The MIF coding region was then subcloned into expression vectors pIN-III-ompA2 and pKP 1500 following the same procedure as described above.

Expression and purification

Step 1: *Fermentation conditions*

200 ml of saturated culture of E. coli YM 109 strain (Miller, H. (1987) Methods in Enzymology 152,145-170), harbouring the MIF expression plasmid pMEX (pKP 1500 with MIF insert) was used to inoculate a 15 l (Chemap LF 7/14/20) bioreactor containing 10 l of sterile LB broth (10g tryptone, 5g yeast extract and 10g of NaCl per l of media) with 10 ml of Silicon 1510 (added as anti foaming agent prior to autoclaving). The seed culture was prepared by inoculating 10 µl of frozen glycerol stock culture into 200 ml of M9 minimal liquid medium, supplemented with 100 mg ampicillin per litter (Sanbrook, J., Maniatis, T. & Fritsch, E. F. Molecular Cloning: A laboratory manual; Cold Spring Harbour Laboratory (1989)) in an 500 ml Erlenmeyer flask and by cultivating at 21°C for 36 hours with agitation on a rotatory shaker (140 rpm). Following seeding, ampicillin (100 mg per l) was added

to the growth medium assepticaliy. Fermentation was carried out with controlied stirring (600 rpm) and aeration (30l/min). The culture was incubated at 37°C for 2,5 to 3 hours (until it reached an A_{600} of 0,5 to 0,1) after which it was induced with IPTG (0,5g/10 l culture) and cultivated for another four hours under the same conditions.

Step II: *Preparation of cell extract*

Four hours after induction with IPTG, the bioreactor was water cooled to 12°C, the culture decanted into glass flasks and immediately chilled on ice. Bacteria from the fermentation broth were harvested by centrifugation at 8000g and 4°C for 20 minutes. A total of 50 grams of cell pellet was suspended in 200 ml of sterile water. The suspension was freeze-thawed three times and then sonicated with one burst (1,5 minute) in a 70 W ultrasonic ice water bath. Following centrifugation at 8000 g for 20 minutes at 4°C to remove cellular debris the supernatant was filtered trough a Millipore low protein binding membrane (0,2 µm) and transferred to a 350 ml stirred ultrafiltration cell (Amicon) equipped with YM2 (2000 mol. wt. cut off) membrane and concentrated five fold at 4°C.

Step III: *Gel filtration chromatography*

A glass column (50 mm diameter) was packed with Sephadex G-50 (bed height 1500 mm) and equilibrated with buffer A (0,1 M Tris buffer pH 7,4; 0,3M NaCl; 1 mM EDTA). The sample (20 ml of the concentrated supernatant from step II) was carefully added on the top of the gel bed and eluted with buffer A mentioned above. Fractions were collected automatically. The flow rate was 42 ml/h and the fraction volume was 14 ml. The total procedure was performed at 4°C and it required approximately 32 hours. The Sephadex G-50 fractions were monitored by absorbance at 280 nm for protein. Some fractions were analysed by SDS-PAGE and IEF where the protein extract of E. coli YM 109 cells, bearing the expression vector without insert, was used as a control. Aliquots of fractions were dialysed by a mini dialysis method (Francky Andrej; personal communication) prior to further analyses by IEF and

SDS-PAGE. Several fractions containing recombinant protein were pooled and concentrated on Amicon concentrator to 150 ml.

Step IV: *Ion exchange chromatography*

CM cellulose which had been previously regenerated according to the manufacturer instructions and suspended in buffer B (10 mM phosphate buffer/pH 6,4) was used to pack the column which was then equilibrated with the same buffer. 75 ml of the concentrated sample (from step III) was dialysed against buffer B and loaded on the CM cellulose column (bed volume 250 ml). After the column was washed to remove unbound protein (until the absorbance at 280 nm was less than 0,05) the protein was eluted with a linear NaCl gradient generated from 500 ml of buffer B and 500 ml of buffer B containing 0,3 M NaCl. The separation was performed at 4°C. The eluate was collected automatically with a flow rate of 19 ml/h. Fractions of 6,3 ml were collected. Elution was monitored in the same way as described above. All fractions from the second peak contained purified recombinant MIF. They were pooled, dialysed against distilled water at 4°C and concentrated to a concentration of 5 mg per ml by ultrafiltration. The purified protein was then stored at minus 70°C. Some samples were lyophilised prior to storage.

Electrophoresis

Protein purity and molecular weight were determined by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) on a Pharmacia Phast System using prefabricated 8 to 25 % gradient polyacrylamide gels and the Sigma SDS 7 molecular weight standard mixture (containing seven proteins in the 14200 to 94000 mol. wt. range). The proteins were electrophoresed and stained with Comassie brilliant blue G-250 according to the manufacturer instructions.

For protein analyses of whole cell lysates, cells were peleted by centrifugation, resuspended in water and mixed with an equal amount of 2X SDS loading buffer, boiled for 5 minutes and sonicated with one burst (1 minute) in an ultrasonic water bath. After centrifugation (Eppendorf microcentrifuge, 14000 rpm, 10 minutes) the supernatant was diluted with 1X loading buffer when necessary and subjected to SDS -PAGE.

Isoelectric focusing (IEF)

An IEF gel (thickness 1 mm) was prepared using 5% T and 5% C, 10% glycerol, 0,45 % ammonium persulphate and 6,66% Pharmalytes (pH 3-10). Prefocusing was carried out at a constant power of 25 W (voltage limit 300 V) at 7°C. After the samples (20-50 µl each), together with standards (pI 3,5 to 9,3 / Pharmacia), were spotted on the gels, the IEF was carried out at constant power of 25 W (voltage limit 1500 V) for approximately 2 hours. The proteins in the gel were fixed by soaking the gel in 20% trichloroacetic acid for 30 min; stained with a solution of 0,2% Coomassie blue G-250 / 45% methanol / 10% acetic acid for 5 min and destained with the same solution without G-250. Alternatively, proteins were focused on a Phast Gel System (Pharmacia) using precast gels, pH range 3 to 9.

Determination of the N-terminal aminoacid sequence

The amino terminal sequence of the recombinant protein was determined by Edman degradation using an automated Applied Biosystems (Foster City, CA) Model 477A pulsed liquid phase protein sequencer with an Model 120A on line PTH amino acid analyser. Sequencing was performed with regular cycle programs and chemicals from the manufacturer.

MIF biological assay

The biological activity of the recombinant human MIF was determined using guinea pig peritoneal macrophages as indicator cells according to the method described by Harrington et al. (Harrington J. T., JR. & Stastny P. (1973), J. Immunol. 110 (3), 752-759).

The percentage of inhibition was derived as follows: % inhibition = 100 - (average migration of test samples/average migration of control samples) X 100. Inhibition of 20% or greater was considered to be significant

Imunohistochemical methods

For immunohistochemical detections of MIF we used commercial kits and we followed the instructions of the manufacturers. We used primary antibodies developed against purified recombinant human MIF.

The above described example serves just for illustration and do not limit the scope of the present invention.

CLAIMS

1. Oligonucleotide primers (their sequence is shown below) and oligonucleotides with other sequences, characterised by the ability to enable amplification of the nucleotide sequence, encoding human MIF by polymerase chain reaction and subsequent cloning of the PCR product in different vectors.

I. : 5' GGATCCGAATTC**ATG**CCGATGTTCATCGTAAACACCA 3'

II.: 5' GTCGACAAGCTT**TTA**GGCGAAGGTGGAGTTGTTCCA 3'

2. The expression vector construct pMEX, characterised by including the below presented nucleotide sequence, encoding human MIF or by including other nucleotide sequences, encoding other proteins which are not different in more than twenty amino acids from the sequence describec below and that all sequences mentioned are integrated into a vector on the way shown on fig. 1.

```
ATG CCG ATG TTC ATC GTA AAC ACC AAC GTG CCC CGC GCC TCC GTG     45
Met Pro Met Phe Ile Val Asn Thr Asn Val Pro Arg Ala Ser Val
                 5                  10                  15

CCG GAC GGG TTC CTC TCC GAG CTC ACC CAG CAG CTG GCG CAG GCC     90
Pro Asp Gly Phe Leu Ser Glu Leu Thr Gln Gln Leu Ala Gln Ala
                20                  25                  30

ACC GGC AAG CCC CCC CAG TAC ATC GCG GTG CAC GTG GTC CCG GAC    135
Thr Gly Lys Pro Pro Gln Tyr Ile Ala Val His Val Val Pro Asp
                35                  40                  45

CAG CTC ATG GCC TTC GGC GGC TCC AGC GAG CCG TGC GCG CTC TGC    180
Gln Leu Met Ala Phe Gly Gly Ser Ser Glu Pro Cys Ala Leu Cys
                50                  55                  60
```

AGC CTG CAC AGC ATC GGC AAG ATC GGC GGC GCG CAG AAC CGC TCC 225
Ser Leu His Ser Ile Gly Lys Ile Gly Gly Ala Gln Asn Arg Ser
65 70 75

TAC AGC AAG CTG CTG TGC GGC CTG CTG GCC GAG CGC CTG CGC ATC 270
Tyr Ser Lys Leu Leu Cys Gly Leu Leu Ala Glu Arg Leu Arg Ile
80 85 90

AGC CCG GAC AGG GTC TAC ATC AAC TAT TAC GAC ATG AAC GCG GCC 315
Ser Pro Asp Arg Val Tyr Ile Asn Tyr Tyr Asp Met Asn Ala Ala
95 100 105

AAC GTG GGC TGG AAC AAC TCC ACC TTC GCC TAA 360
Asn Val Gly Trp Asn Asn Ser Thr Phe Ala *
110 115

3. The procedure of recombinant *E. coli* cultivation characterised by such a combination of expression vector, bacterial host strain and cultivation conditions that recombinant MIF is produced in soluble form in the heterologous system of *Escherichia coli*

4. The procedure of isolation and purification of recombinant MIF characterised by the combination of following methods:

a.) the method for bacterial cell disruption by freezing and thawing only

b.) two step purification protocol where gel filtration and ion exchange chromatography are included

c.) the method for MIF quantification/estimation during the purification procedure on the basis of IEF, SDS-PAGE, Western blotting or ELISA analysis

5. The below presented nucleotide sequence characterised by encoding human MIF in the epithelial cells of different tissues, like in epithelial cells of the human uterus endometrium.

ATG CCG ATG TTC ATC GTA AAC ACC AAC GTG CCC CGC GCC TCC GTG 45

CCG GAC GGG TTC CTC TCC GAG CTC ACC CAG CAG CTG GCG CAG GCC 90

ACC GGC AAG CCC CCC CAG TAC ATC GCG GTG CAC GTG GTC CCG GAC 135

CAG CTC ATG GCC TTC GGC GGC TCC AGC GAG CCG TGC GCG CTC TGC 180

AGC CTG CAC AGC ATC GGC AAG ATC GGC GGC GCG CAG AAC CGC TCC 225

TAC AGC AAG CTG CTG TGC GGC CTG CTG GCC GAG CGC CTG CGC ATC 270

AGC CCG GAC AGG GTC TAC ATC AAC TAT TAC GAC ATG AAC GCG GCC 315

AAT GTG GGC TGG AAC AAC TCC ACC TTC GCC TAA 360

6. The recombinant protein, characterised by the below presented amino acid sequence or other amino acid sequences that do not differ in more than twenty amino acids from the sequence shown below and which is obtained on the way described (related to the points 3 and 4) or on any other similar manner and its therapeutic, diagnostic and any other uses which are not for scientific purposes only.

Met	Pro	Met	Phe	Ile	Val	Asn	Thr	Asn	Val	Pro	Arg	Ala	Ser	Val
				5					10					15
Pro	Asp	Gly	Phe	Leu	Ser	Glu	Leu	Thr	Gln	Gln	Leu	Ala	Gln	Ala
				20					25					30
Thr	Gly	Lys	Pro	Pro	Gln	Tyr	Ile	Ala	Val	His	Val	Val	Pro	Asp
				35					40					45
Gln	Leu	Met	Ala	Phe	Gly	Gly	Ser	Ser	Glu	Pro	Cys	Ala	Leu	Cys
				50					55					60
Ser	Leu	His	Ser	Ile	Gly	Lys	Ile	Gly	Gly	Ala	Gln	Asn	Arg	Ser
				65					70					75
Tyr	Ser	Lys	Leu	Leu	Cys	Gly	Leu	Leu	Ala	Glu	Arg	Leu	Arg	Ile
				80					85					90
Ser	Pro	Asp	Arg	Val	Tyr	Ile	Asn	Tyr	Tyr	Asp	Met	Asn	Ala	Ala
				95					100					105
Asn	Val	Gly	Trp	Asn	Asn	Ser	Thr	Phe	Ala					
				110					115					

7. Antibodies (policlonal or monoclonal) which are prepared by use of the recombinant MIF (related to the point 6) and their therapeutic, diagnostic and any other uses that are not for scientific purposes only.

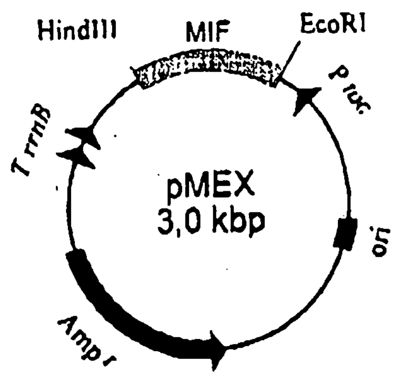


Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/SI 95/00022

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/19 C07K14/52 C07K16/24 A61K38/19 A61K39/395
C12N15/11 C12N15/70 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,90 11301 (GENETICS INSTITUTE, INC.) 4 October 1990 see page 37, line 18 - page 38, line 15; claims; table I ---	5,6 2,3
Y	PROTEIN ENGINEERING, vol. 1, no. 4, 1987 ENGLAND GB, pages 327-332, TAKEYOSHI MIKI ET AL 'Construction of a plasmid vector for the regulatable high level expression of eukaryotic genes in Escherichia coli : an application to overproduction of chicken lysozyme' cited in the application see page 328, right column; figure 1 --- -/--	2,3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 1996	14.03.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Le Cornec, N

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/SI 95/00022

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 86, October 1989 WASHINGTON US, pages 7522-7526, W. Y. WEISER ET AL 'Molecular cloning of a cDNA encoding a human Macrophage Migration Inhibitory Factor' cited in the application see the whole document	5,6
Y		2,3

X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, November 1993 WASHINGTON US, pages 10056-10060, TOSHIFUMI MIKAYAMA ET AL 'Molecular cloning and functional expression of a cDNA encoding glycosylation-inhibiting factor' see the whole document	1-7

A	WO,A,92 19727 (NATIONAL INSTITUTES OF HEALTH) 12 November 1992 see page 11 - page 13	1,5

X	JOURNAL OF MOLECULAR BIOLOGY, vol. 235, no. 3, March 1994 pages 1141-1143, MAMORU SUZUKI ET AL 'Crystallization and a preliminary X-ray diffraction stydy of Macrophage Migration Inhibitory Factor from human Lymphocytes' cited in the application see page 1142	1,3,5,6
Y		2

X	GENOMICS, vol. 19, no. 1, 1 January 1994 pages 48-51, V. PARALKAR ET AL 'Cloning the human gene for Macrophage Migration Inhibitory Factor (MIF)' see the whole document	1

P,X	BIOCHEMISTRY, vol. 33, no. 47, 29 November 1994 EASTON, PA US, pages 14144-14155, J. BERNHAGEN ET AL 'Purification ,bioactivity, and secondary structure analysis of mouse and human Macrophage Migration Inhibitory Factor (MIF)' see page 14145, right column, line 14 - page 14146, left column, line 30 see page 14147, right column	1,3-6

1

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/SI 95/00022

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,94 26307 (THE PICOWER INSTITUTE FOR MEDICAL RESEARCH) 24 November 1994 see page 64 - page 67, line 11 see example 7 see page 54, line 30 - page 58, line 3	1,3-7
Y		2

X	NATURE, vol. 365, 21 October 1993 LONDON GB, pages 756-759, J. BERNHAGEN ET AL 'MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia' cited in the application see page 757	6,7
A		1,4,5
Y		2,3

1